PEGylated Precision Segments Based on Sequence-Defined Thiolactone Oligomers

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Abstract

A straightforward access route to multifunctional block copolymers, com- bining a poly(ethylene glycol) (PEG) block and a monodisperse segment with discrete monomer sequence based on thiolactone chemistry, is described. Exploiting an inverse conjugation strategy on a PEG preloaded poly(styrene) synthesis resin enables the convenient introduction of a predefined PEG-block at the α-terminus of thiolactone-based sequence-defined oligomers. Reaction conditions for the stepwise, submonomer synthesis at polar solid supports are optimized, using sequential synthesis on a model resin that enables to isolate and determine the purity of the oligomer segments by liquid chroma- tography-electrospray ionization mass spectrometry analysis. The reaction conditions are used to synthesize PEGylated 5*mer* precision polymers with defined monomer sequence in good yields and high purity to offer an inter- esting platform of macromolecules with potential for biomedical applications.

1. Introduction

Intrigued by nature's sophisticated biomacromolecules, which— if required—are defined down to the monomer sequence level, polymer chemists directed the attention to rebuild informa- tion rich macromolecules synthetically, exploiting initially bio- macromolecule platforms.[1–7] In recent years, new chemistries were established to achieve monomer sequence control along synthetic polymer chains with fully synthetic monomer alpha- bets, aiming to implement information or precisely program structure–property relationships.[8–17] Among various routes for the preparation of monodisperse sequence-defined macro- molecules,[18] solid-phase synthesis (SPS) proved to provide a useful platform, enabling to expand the concepts of Merrifield's sequential peptide synthesis[19] toward new chemistries.[14,16,20] Du Prez and co-workers have recently introduced a synthesis route to obtain sequence-defined multifunctional oligomers by exploiting thiolactone chemistry via two step iterative SPS.[13] In the first step, thi- olactone moieties are anchored onto the support via urea linkage by reacting amino groups of the resin with α-isocyanate-γ- thiolactone (Tla–NCO). Subsequently the resin bound thiolactone is opened by ami- nolysis with an amino alcohol, releasing thiol functionalities to react cleanly via classical Michael addition with an acrylate. The latter enables to introduce a variety of functionalities by exploiting the set of commercially available or easily synthe- sizable monomers. After this reaction step, the reaction circle of the sequential submonomer synthesis starts again by attaching the isocyanate group of Tla– NCO to the remaining alcohol group that was introduced by the amino alcohol.

In recent decades, poly(ethylene glycol) (PEG) conjugation to, e.g., peptides or proteins[21–25] developed to a generic tool to positively modulate a broad range of properties such as for instance improving solubility, bioavailability, and reducing bio-clearance, but also influencing thermal stability, aggregation propensity and enzymatic processability.[23,25] As a result, a rich set of applications of PEG-bioconjugates is nowadays available reaching from biomedical to materials sciences.[26–35] Taking into account the impact of PEG conjugation—referred to as PEGylation—, it is expected to offer valuable opportunities also in the world of precision polymers in general and specifically with those multifunctional thiolactone-based sequence-defined oligomers. Compared to peptide-based bioconjugates, advan- tages of PEGylated constructs with fully synthetic precision segments are evident due to the lack of a potential inherent risk of toxicity or/and immunogenicity. This provides peptide-mimetic polymers based on precision macromolecular seg- ments with interesting application potential including, e.g., drug solubilizers or transporters, as well as compatibilizers and antifouling coatings, as have been most successfully demonstrated already by peptide systems.[36–43] For PEG-peptide con- jugate synthesis, a set of capable PEGylation strategies have been developed, which include coupling of end functional PEG at selectively addressable functionalities on a peptide either in solution or at solid supports.[44–48] However, the inverse conju- gation strategy proved to be most straightforward and useful for materials science applications. This starts from the syn- thesis of the peptide segment in a stepwise manner at a sup-ported PEG-block, which is preloaded on the PAP-synthesis resin (PEG attached poly(styrene) resin).[49] After synthesis, the PEG-peptide conjugate is liberated from the support and often the fully deprotected conjugate exhibits sufficient quality and is readily acceptable for the use in materials science applications. Frequently, the inverse conjugation on PAP resins improves

both the coupling rates and purities for peptide synthesis by reducing peptide/peptide and peptide/resin aggregation. However, the change in polarity of the resin composed of about 85 wt% PEG with its capacity to tightly bind water imposes challenges for the sequential submonomer synthesis using thi- olactone/Michael addition chemistry.

Here, we present the adaptation of thiolactone-based precision polymer synthesis protocols from hydrophobic poly(styrene) resins with acid labile 2-chlorotrityl linkers toward polar PAP resins linked with benzyl ethers, requiring strong acidic conditions to liberate the product. This enables ease of synthesis of PEGylated thiolactone-based precision polymer segments, exhibiting a monodisperse, sequence-defined oli- gomer segment by inverse conjugation strategies.

2. Experimental Section

A detailed description on methods and synthesis procedures as well as compound analytics is provided in the Supporting Information section.

3. Results and Discussions

To investigate the straightforward and flexible strategy toward PEGylated thiolactone-based oligomers an initial synthesis has been conducted. This applied conditions of the standard primary protocol as reported by Du Prez et al. for the synthesis of thiolactone-based sequence-defined oligomers on hydro- phobic poly(styrene) (PS) resins. However, instead of the PS supports with 2-chlorotrityl linkers, the inverse conjugation of thiolactone-based oligomers was conducted on a commercially available PAP resin, preloaded with PEG of $M_{n,PEO} \approx 3200$ and anchored via a benzyl ether linker. Following the standard pri- mary protocol Tla–NCO coupling to the resin required 1 h treat- ment with 10 equiv. Tla–NCO in chloroform and 0.025 equiv. dibutyltin dilaurate relative to the resin-bound alcohol-units to introduce the first Tla moieties to the resin. Subsequently, chain extension involved 15 min reaction of the Tla carrying resin with 10 equiv. of 2-amino ethanol and 20 equiv. of the benzyl acrylate (or any other acrylate) as Michael acceptor of choice to perform Tla ring opening and Michael addition in a single pot reaction. After two iterative submonomer reaction cycles, the formation of a thiolactone-based sequence-defined oligomer-PEG conjugate with two repeats, presenting benzyl ester moie- ties in the side chains was expected. In contrast to the reported procedure on PS resins with acid labile 2-chlorotrityl linkers, the solid supported PEG-oligomers could not be released from the support by 5 min treatment with 4% trifluoroacetic acid (TFA) in dichloromethane, but required 100% TFA for 3 h to be liberated. The PEG-oligomers could be isolated by precipitation and analysis via Matrix Assisted Laser Desorption Ionization–Time of Flight (MALDI-TOF) mass spectrometer (**Figure 1**) indicated the presence of the target product as a homologous row with 44 Da distances corresponding to the mass of a PEG reple- tion unit. The overall masses of the signals could be assigned to the expected PEGylated dimer structures with ±0.1 Da accuracy. Interestingly, no hydrolysis products could be detected, proving the robustness of the backbone of the thiolactone-based sequence-defined oligomers against acid hydrolysis, even under harsh TFA conditions. Moreover, the used ester groups present in the side chains are also hydrolytically stable under the given conditions. However, this depends on the ester side chains that will be introduced via the chosen acrylates.

However, under the given synthesis conditions also consider- able side product formation is evident (Figure 1). Most obvious are a set of deletion sequences, which are resulting in a second homologous row, where the overall masses can be assigned with ±0.1 Da accuracy to PEG-blocks with only one repeat of the thiolactone-based unit including a benzyl acrylate side chain.

Analysis of the intermediate products after completion of the first submonomer reaction cycle (Tla–NCO coupling, aminolysis, and Michael addition) indicated evidence for the presence of non-ring-opened Tla moieties, suggesting inefficient aminolysis to be the origin for the occurrence of the deletion sequence (data not shown). As an identical acrylate was used for both sequence positions, it is likely that incomplete aminolysis of the Tla moiety occurring during submonomer synthesis cycle one is fully completed during aminolysis step of submonomer syn- thesis cycle two. As a result this will lead to the formation of a classical deletion sequence as it has been observed. In contrast to standard PS resins, the PAP resin is pronounced hydrophilic and well optimized for polar aprotic solvents used in peptide synthesis such as *N*,*N*-dimethyl-formamide (DMF) or *N*-methyl-2-pyrrolidon (NMP). However, $CHCl_3$ was found to be the most suitable solvent for the aminolysis of supported Tla. While the PAP resin swells even better in chlorinated compared to amidic solvents, $CHCl_3$ might be less capable of reducing oligomer/oligomer as well as oligomer/resin contacts. This could lead to on- resin aggregation, reducing the availability of the terminal func- tionalities that might result in a less effective Tla aminolysis.

The presence of deletion sequences to occur on a polar synthesis resin already at such low sequence length makes the adaptation of the initially developed protocol for the PS resins mandatory. However, the PAP resin seems not to be ideal for this, as the PEG-conjugate products can be analyzed only by MALDI-TOF, reducing ease and accuracy of analysis as minor side products might overlap with mass signals of the homolo- gous row. Taking this analytical issue into account, the protocol development was performed on a hydrophilic TentaGelSRAM resin with rink amide linker, which chemically corresponds very closely to the PAP resin. Similarly to the PAP resin, the TentaGelSRAM resin exhibits a 1% cross-linked PS microgel core with grafted PEG chains of about $M_n = 3000$ g mol^{-1}. How- ever, the linker is not present between PS and PEG enabling liberation of PEG-oligomer conjugates, but between PEG and the thiolactone-based oligomer that will be synthesized. This enables to cleave not the entire PEG-conjugates but only the precision segment, to be analyzed by ultraperformance liquid chromatography-electrospray ionization-mass spectrometry (UPLC-ESI-MS) with high accuracy.

Protocol comparison and protocol adaptation was performed on a polar TentaGelSRAM resin (cf. **Figure 2**). Employing the primary protocol developed for the thiolactone-based sequence-defined oligomers on hydrophobic PS resins, con- firmed the inherent difficulties to realize high purity and clean reactions. After two iterative submonomer reaction cycles with benzyl acrylate as Michael acceptor, the resulting dimers were liberated from the support to be isolated by precipitation. UPLC-ESI-MS analysis of the product confirms what has been suggested from the analysis of the PEG-conjugate by providing evidence of the target dimer at retention time t_R = 3.3 min with 50% purity according to the elution trace of the UV-vis detector at 210 nm. Besides this, a deletion sequence, showing only one repeat of the thiolactone-based unit could be found at t_R = 1.9 min (14%). This indicated a noncomplete thiolactone aminolysis in the first submonomer synthesis cycle, which has been completed during aminolysis and Michael addition at the second submonomer synthesis cycle (cf. Figure S6, Supporting Information). As expected, no difficulties occur in the Michael addition step that seems to run cleanly in the polar environ- ment of the SRAM resin. Careful analysis of the product mix- ture indicated further minor side products including oxidation of the thioether side chain to sulfoxide and TFA ester formation (cf. Figure S6, Supporting Information). Obviously, both resins TentaGelSRAM and PAP behave rather comparable during the synthesis of thiolactone-based oligomers, making TentaGelSRAM most suitable to adapt the reaction conditions for inverse conjugation on a PAP resin. The side product analysis located the difficulties to occur par- ticularly in the aminolysis step of the supported Tla species, resulting in an incompletion of the second part of the sub- monomer synthesis. Moreover, some sequence dependency was observed indicating that aminolysis rates might depend on the neighboring residues (data not shown). Thus, in order to force conversion of this most critical step to completion, the reaction time was extended from 15 to 30 min and this step was repeated three times to ensure always quantitative conversion independent of the sequence.

No significant indications could be found for an incomplete Tla–NCO coupling step as first part of the submonomer synthesis. However, the reaction of isocyanate and resin bound alcohol is in clear competition with isocyanate hydrolysis by reaction with water. Indeed, the known properties of PEG to strongly bind water, make isocyanate hydrolysis more likely to occur at the polar PAP versus the hydrophobic PS resins. To avoid inefficient Tla–NCO coupling steps, the standard pro- tocol was further adjusted by doubling the equivalences used in the original procedure, ensuring the completion within the 1 h reaction time. The newly adjusted protocol has been applied to synthesize a model oligomer on TentaGelSRAM resin, prior to inverse conjugation at a PAP resin (Figure 2). To consolidate the synthesis protocol, a pentamer consisting of five iterative submonomer additions was targeted resulting in a thiolactone-based oligomer with a specific sequence in side chain functionalities (1. benzyl, 2. benzyl, 3. isobutyl, 4. benzyl, 5. benzyl). Following the adapted protocol, the sequence- defined oligomer was assembled within 2–2.5 h overall cycle times per submonomer unit, including careful washing steps. The crude product was liberated from the synthesis resin and could be isolated by precipitation in ether to be analyzed by UPLC-ESI-MS (Figure 2). Compared to the primary protocol for PS-resin synthesis, a reduced number of different species are obvious in the chromatogram, indicating cleaner reactions to occur. The analysis shows only two distinct species, which after assignment result from the same oligomer that has been targeted. Whereas the signal at t_R= 2.5 min (25%) could be cor- related to the product with full length sequence with ±0.1 Da accuracy ([M(1–5)+H]$^+$). The compound eluting at t_R = 3.6 min and 1.7–2.0 min relates with 64% to the TFA ester of the full length sequence that could be assigned with ±0.1 Da accuracy ([M(1–5)+Na+TFA-H_2O]$^+$). TFA ester adducts are well known in solid phase supported peptide synthesis and can occur under TFA conditions with alcohol functionalities, e.g., of serine. The TFA ester rapidly hydrolyses during dialysis, making this side reaction to a nonpermanent modification that is removable under standard purification conditions.

Ultimate proof of transferability of the adapted synthesis protocol for polar, PEG based resins was demonstrated by performing a pentamer synthesis on a PAP resin with preloaded PEG of M_n = 3200 g mol^{-1} (cf. **Figure 3** and Supporting Information). For proof of principle, a model 5*mer* sequence has been chosen, which was composed of five different residues. The sequence of the thiolactone-based oligomer segment was not addressing a particular function. However, the targeted oligomer illustrated the flexibility of the submonomer synthesis by incorporating a small set of pendent ester functionalities, covering aromatic, polar/nonionic, unipolar, and sterically demanding hydrophobic ester properties. The submonomer synthesis was performed with an overall cycle time of 2 h including the required washing steps in-between the reaction steps. After completion of the synthesis, the PEG-conjugate was liberated from the support via TFA treatments and the product could be isolated by precipitation to perform MALDI-TOF MS analysis. The obtained MALDI-TOF MS spectra for the thiolactone-based oligomer-PEG conjugate indicated no evidences for deletion sequences. The overall masses of each signal could be assigned to the TFA adduct of the expected product with ±1.3 Da accuracy. Furthermore, additional peak sets appeared with a mass difference of 16 Da, showing the partial oxidation of one thioether side chain to sulfoxide. Probably the oxidation occurs during the acidic cleavage of the PEG-conjugate from the resin. Thioether oxidation is well known in peptide chemistry as oxidation of side chain functionality of methionine to sul- foxide can frequently be observed as side reaction during acidic cleavage. The oxidation can be effectively suppressed by the addition of thioether scavengers, which have not been added in this study, as the low molecular weight compounds reduced the resolution achievable during MALDI-TOF-MS analysis.

4. Conclusion

A protocol toward synthesis of thiolactone-based sequence- defined oligomers conjugated to poly(ethylene glycol) (PEG-

conjugates) was adapted based on the primary protocol that has been previously developed for the synthesis of thiolactone-based oligomers on hydrophobic poly(styrene) resins. The sub- monomer protocol adapted for polar synthesis resins accounts for improved Tla–NCO coupling and Tla aminolysis to result in significantly reduced side reactions and higher purity. Applying the iterative synthesis on a TentaGel PAP resin, straightforward access to PEG-oligomers is enabled, providing conjugates with high purity and defined monomer sequence in the monodis- perse thiolactone-based oligomer segments. The reaction con- ditions for the submonomer synthesis using polar synthesis resins were optimized, by making use of sequential synthesis via thiolactone chemistry on a model resin that allows for the accurate determination of potential side reactions by UPLC- ESI-MS analysis. The reaction conditions have been used to synthesize conveniently PEGylated 5mer precision polymers with a defined monomer sequence in good yields and suitable purity. These conjugates might offer an interesting platform of macromolecules with potentials for biomedical applications. For instance, the sequences in the thiolactone-based oligomers might be tailored to specifically host drug entities or to adhere to certain material surfaces, leading to precisely adjustable drug solubilizers or surface specific anti-fouling coatings. Certainly it appears to be an interesting strategy to extract sequence information from peptide based systems, where screening, sequencing, and computational modeling is routine in many laboratories.[50]

Keywords

PEG conjugates, PEGylation, precision polymers, sequence-defined oligomers, solid phase synthesis

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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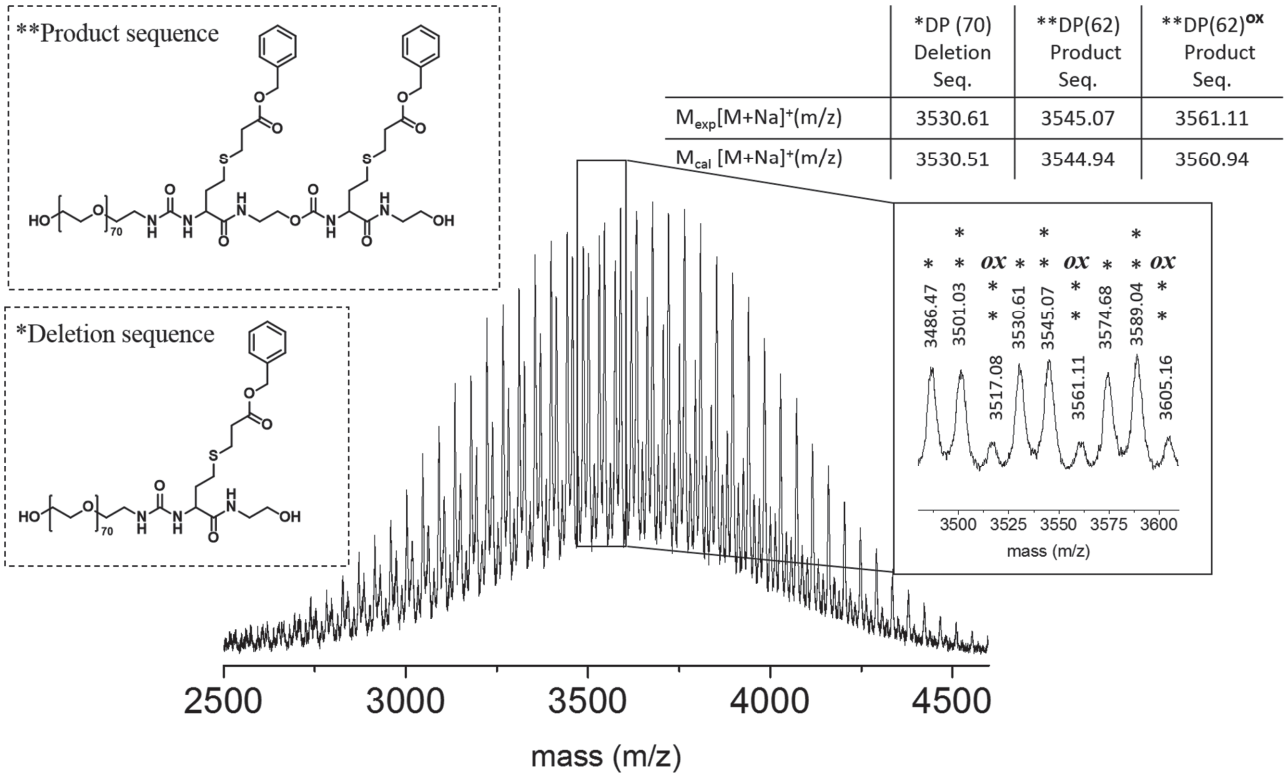


Figure 1. MALDI-TOF mass spectrometry analysis of an inverse conjugation of thiolactone-based oligomers on a PAP resin, leading to directly access PEG-precision oligomer conjugates by following the primary synthesis protocol that was optimized for PS resins (Conditions: 10 equiv. Tla–NCO in $CHCl_3$ and 0.025 equiv. dibutyltin dilaurate relative to the resin-bound alcohol-units in 1 h at r.t. as for chain extension step, 10 equiv. of 2-amino ethanol and 20 equiv. of acrylate of choice in $CHCl_3$ for 15 min (repeated two times) as for one-pot double modification of resin-bound Tla).

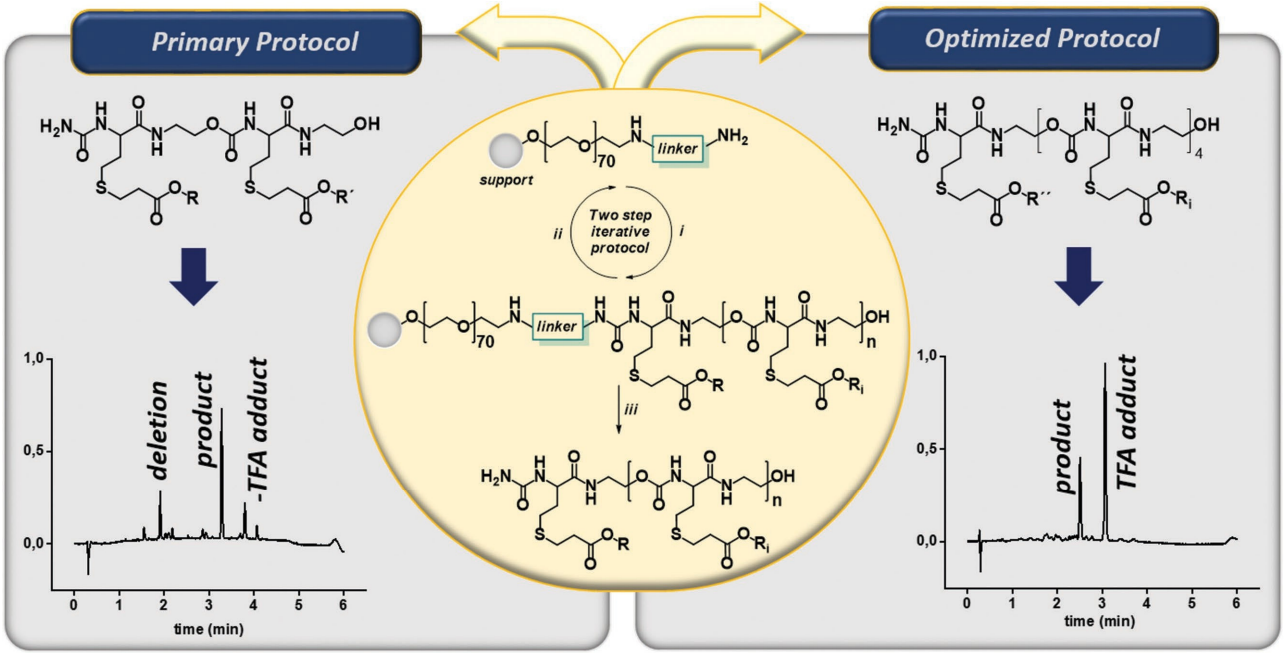
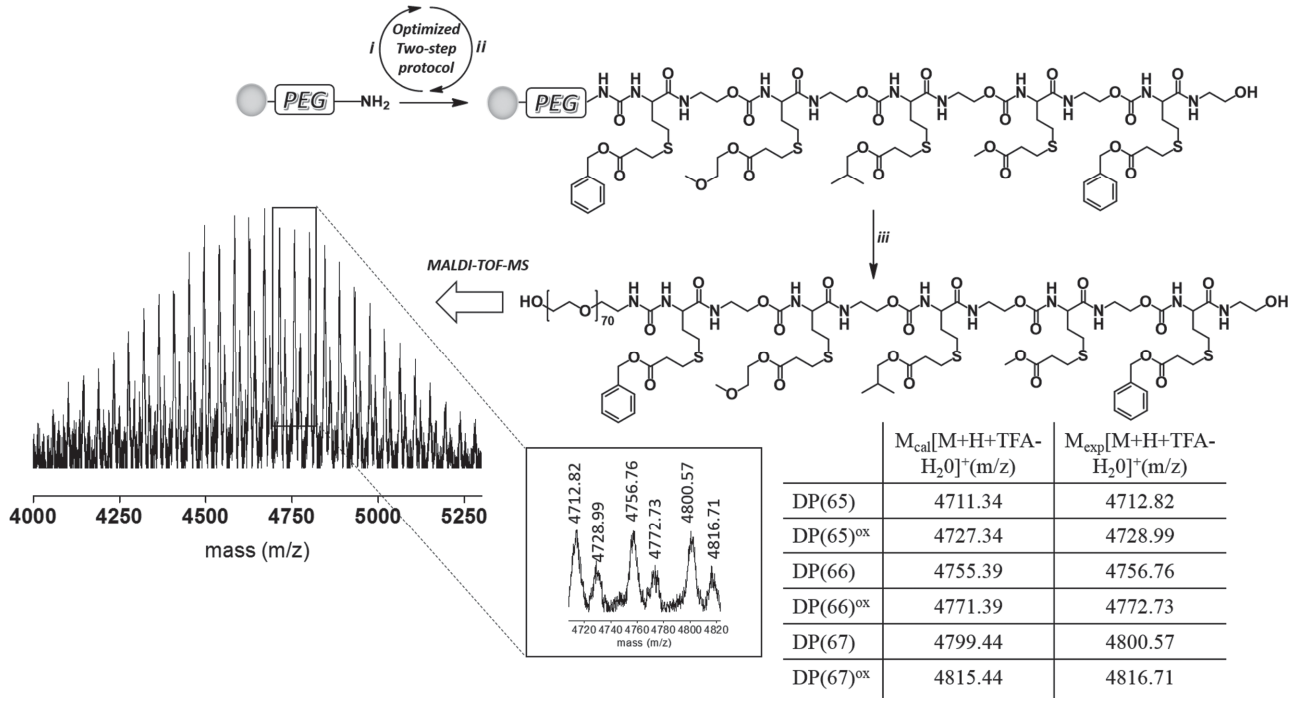


Figure 2. Thiolactone-based sequence-defined oligomer synthesis on SRAM resin comparing the initial primary protocol for PS-resin-based synthesis (left) and the adapted synthesis procedure (right) (Conditions: Primary protocol (left): i) 10 equiv. Tla–NCO in CHCl3 and 0.025 equiv. dibutyltin dilaurate relative to the resin-bound alcohol-units in 1 h at r.t., ii) 10 equiv. of 2-amino ethanol and 20 equiv. of acrylate of choice in CHCl3 for 15 min (repeated twice), iii) 100% TFA at r.t overnight; adapted protocol (right): i) 20 equiv. Tla–NCO and 0.025 equiv. dibutyltin dilaurate relative to the resin-bound alcohol-units in CHCl3 for 1 h at r.t. (repeated three times), ii) 20 equiv. of 2-amino ethanol and 40 equiv. of acrylate of choice in CHCl3 for 30 min (repeated twice), iii) 100% TFA at r.t overnight.



	$M_{cal}[M+H+TFA-H_20]^+(m/z)$	$M_{exp}[M+H+TFA-H_20]^+(m/z)$
DP(65)	4711.34	4712.82
DP(65)^{ox}	4727.34	4728.99
DP(66)	4755.39	4756.76
DP(66)^{ox}	4771.39	4772.73
DP(67)	4799.44	4800.57
DP(67)^{ox}	4815.44	4816.71

Figure 3. Direct synthesis of a thiolactone-based oligomer-PEG conjugate by inverse conjugation at a PAP synthesis resin, applying the adapted pro- tocol and MALDI-TOF mass spectrometry analysis of the obtained product (Conditions: i) 20 equiv. Tla–NCO in CHCl3 for 1 h at r.t., ii) 20 equiv. of 2-amino ethanol and 40 equiv. of acrylate of choice in CHCl3 for 30 min (repeated three times), and iii) TFA cleavage in 100% TFA, overnight).